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Final summary of results:

The Geochemical Rate/RNA Integration Study (GRIST) project sought to correlate biogeochemical flux rates with measurements of gene expression and mRNA abundance to demonstrate the application of molecular approaches to estimate the presence and magnitude of a suite of biogeochemical processes. The study was headed by Lee Kerkhoff of Rutgers University. In this component of the GRIST study, we characterized ambient nutrient concentrations and measured uptake rates for dissolved inorganic nitrogen (DIN, ammonium, nitrate and nitrite) and dissolved organic nitrogen (urea and dissolved free amino acids) during two diel studies at the Long-Term Ecosystem Observatory (LEO-15) on the New Jersey continental shelf.

We found that surface waters were practically devoid (<4%) of DIN forms; urea was the most abundant nitrogen substrate measured (Figure 1). In the bottom waters, nitrogen substrates were primarily inorganic (mean = 65%).

Size-fractionated uptake rates were calculated for the total microbial (>0.2μm), total phytoplankton (>GF/F), and larger phytoplankton (>3μm or >5μm) fractions. Urea dominated uptake by all size fractions at the surface, and ammonium uptake was greatest in the bottom layer (Figure 2). Size-fractionated uptake results suggest that urea uptake was mostly by phytoplankton >3μm, while sequence analysis of *ure*C genes present in surface waters indicate that members of the *Cyanobacteria* and alpha *Proteobacteria* were the predominant urea-utilizing microbes <3μm. In addition, bacteria were responsible for 20-49% of the size-fractionated ammonium and nitrate uptake in surface samples and 36-93% in bottom samples. These results suggest that bacterial competition with phytoplankton for available DIN may force phytoplankton to rely more on DON sources such as urea to meet their cellular nitrogen demands.

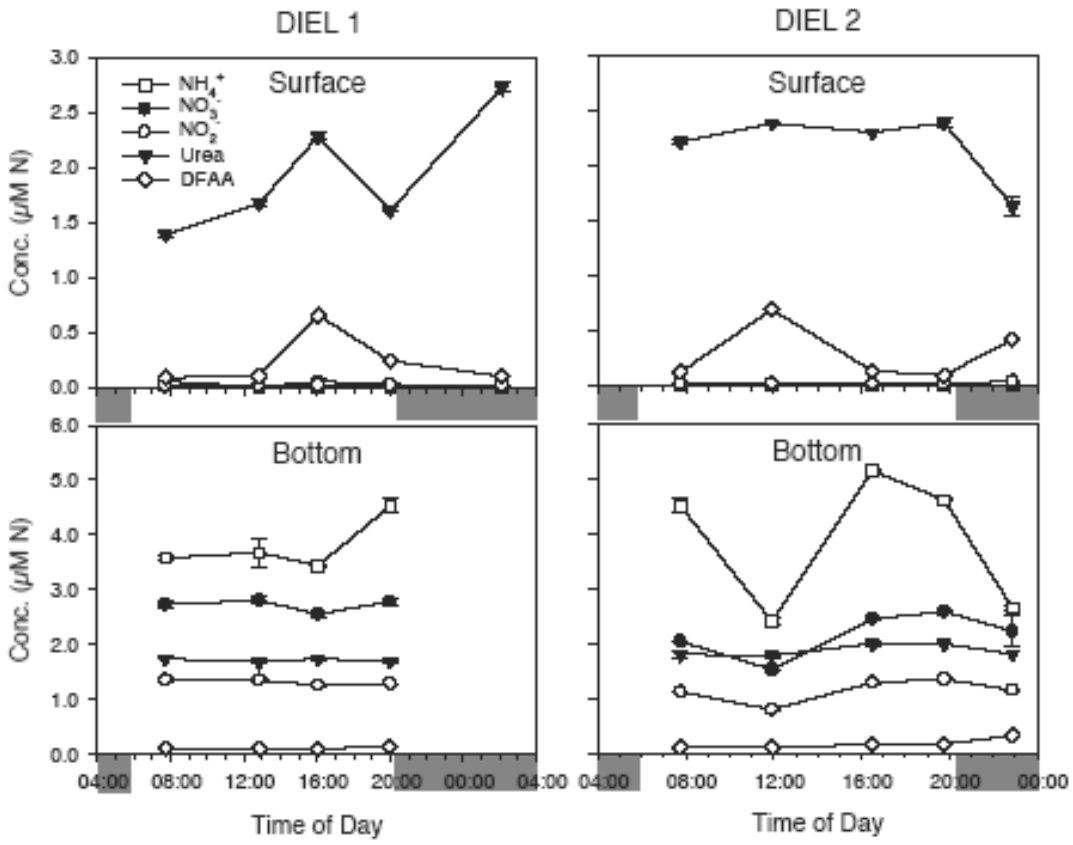

Figure 1. Dissolved nutrient concentrations measured in surface and bottom waters at LEO-15 during two diel experiments: Diel 1, 20-21 July 2002 and Diel 2, 22-23 July 2002. Note the two-fold increase in scale between surface and bottom. Error bars denote ± 1 SD of the mean. Shaded bars indicate dark periods. Surface concentrations of NH_4^+, NO_3^-, and NO_2^- were typically below detection (0.05, 0.03, 0.03 μM, respectively) and thus are not distinguishable from zero.

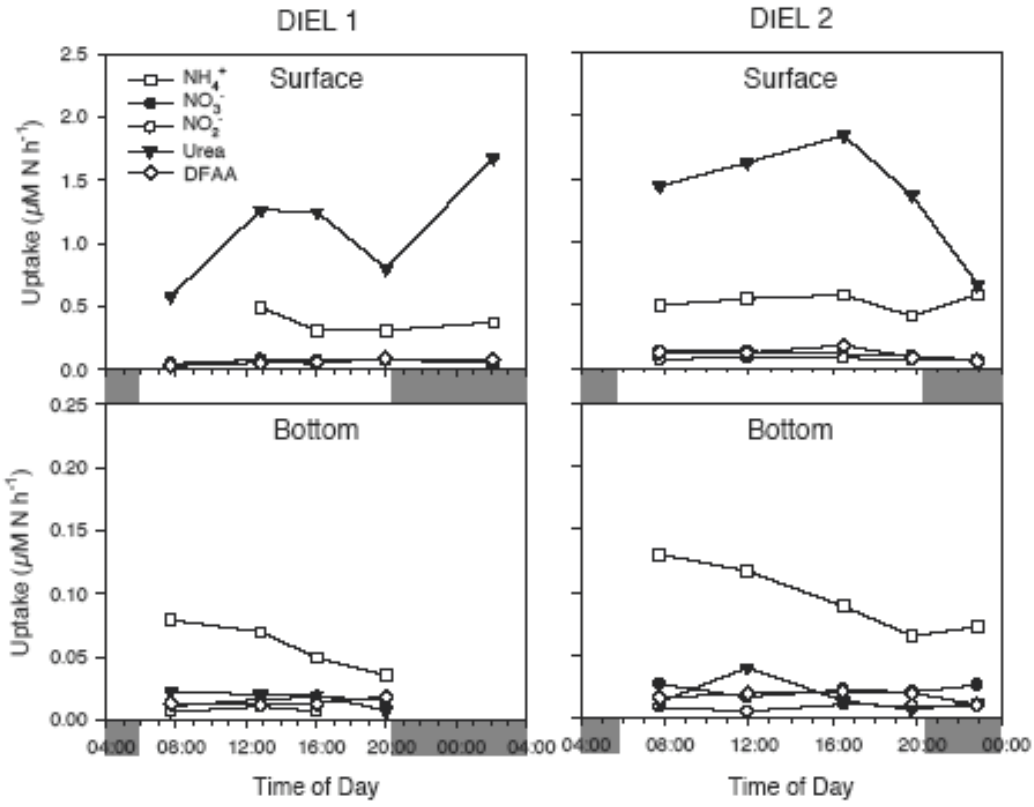

Figure 2. Absolute nitrogen uptake rates (ρ: μM N h^{-1}) measured using GF/F filters from two diel experiments at LEO-15 in July 2002. Note the ten-fold decrease in scale between surface and bottom samples. Shaded bars indicate dark periods

Products delivered:

Bradley, P. B. and D. A. Bronk. 2006. Microbial Nitrogen Use in the Mid-Atlantic Bight Upwelling Region: A Comparison of Size-Fractionation and Flow Cytometric Sorting Approaches. Talk presented at the ASLO/AGU Ocean Sciences meeting, Honolulu, HI.
Bradley, P. and D. A. Bronk. 2005. Use of flow cytometric sorting in the measurement of autotrophic nitrogen utilization. Talk presented at the ASLO meeting in Salt Lake City.

Bronk, D. A., Bradley, P., Sanderson, M, and Frischer, M. 2002. Diel study of inorganic and organic nitrogen utilization at LEO-15. Poster at the BI-OMP conference in St. Petersburg, FL. December 2002.

Products in preparation:

This grant partially funded the work of Paul Bradley, a PhD student at VIMS. Paul has been doing a Knauss Fellowship this year but will be returning to VIMS in February 2008 to complete his dissertation and publish several manuscripts – one of his chapters that is being written as a manuscript focuses on the GRIST results.

Bradley, P., Bronk, D. A., M. E. Frischer, J. E. Brofft, M. G. Booth, M. P. Sanderson, and L. J. Kerkhof. Influence of summer stratification on nitrogen uptake by phytoplankton and heterotrophic bacteria in a Mid-Atlantic Bight upwelling region. In preparation for Estuarine Coastal and Shelf Science (attached).

Influence of summer stratification on inorganic and organic nitrogen uptake by a microbial community in a Mid-Atlantic Bight upwelling region

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Running head: Microbial DIN and DON uptake at LEO-15

ABSTRACT: Little is known about the relative importance of inorganic versus organic nitrogen (N) sources to plankton N nutrition in marine systems. To address this, we conducted two diel studies in the upwelling region of the Mid-Atlantic Bight at the Long-term Ecosystem Observatory LEO-15 off southern New Jersey. Ambient nutrients were characterized and uptake rates measured for dissolved inorganic N (DIN: ammonium, NH_4^+; nitrate, NO_3^-; and nitrite, NO_2^-) and dissolved organic N (DON: urea and dissolved free amino acids) substrates in samples taken approximately every four hours from the surface and bottom mixed layers. Size-fractionated uptake rates were calculated for the total microbial (>0.2 μm), total phytoplankton (>GF/F), and larger phytoplankton (>3 or >5 μm) fractions. The surface total dissolved N (TDN) pool was virtually devoid of DIN, with DON representing > 99% of TDN concentrations. The bottom-water TDN pool, however, was divided evenly between NH_4^+, NO_3^-, and DON. Urea dominated uptake by all size fractions at the surface, while NH_4^+ uptake was greatest in the bottom layer. Size-fractionated uptake results suggest that urea uptake was mostly by phytoplankton >3 μm; sequence analysis of *ure*C genes present in surface waters indicates that members of the *Cyanobacteria* and alpha *Proteobacteria* were the predominant urea-utilizing microbes <3 μm. In addition, the bacterial size fraction was responsible for 20-49% of the size-fractionated NH_4^+ and NO_3^- uptake in surface samples and 36-93% in bottom samples. Based on these results, we hypothesize that bacterial competition with phytoplankton for available DIN may force phytoplankton to rely more on DON sources, such as urea, to meet their cellular N demands.

KEY WORDS: Dissolved organic nitrogen; Dissolved inorganic nitrogen; Uptake rates; LEO-15; Phytoplankton; Bacteria; Mid-Atlantic Bight; Continental shelf

INTRODUCTION

Continental shelf ecosystems are characterized by highly variable, often transient environmental conditions, such as wind-driven upwelling, that dramatically alter the supply of nitrogen (N) and other nutrients to the microbial community. Since coastal waters are often N-limited, such that the bioavailability and rate of supply of ambient N ultimately control primary productivity and consequently ecosystem trophic state (Ryther & Dunstan 1971, Eppley & Peterson 1979, Howarth 1988, Codispoti 1989), these intermittent physical events can largely determine ecosystem productivity. Nitrogen sources to coastal waters include terrestrial inputs, atmospheric deposition, biotic water column processes, upwelling, and sediment remineralization (Capone 2000). Of these various inputs, coastal upwelling represents a significant, albeit ephemeral, source of new N to the surface water during summer months, and often stimulates diatom-dominated phytoplankton blooms (Kokkinakis & Wheeler 1987).

The diverse nature of N sources to coastal waters is reflected in the widely varying composition and biolability of the ambient N pool, which includes both inorganic and organic forms. Dissolved inorganic N (DIN) consists of ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-). The dissolved organic N (DON) pool, however, is a complex mixture of various compounds, including urea, amino sugars, dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA: oligopeptides, proteins), nucleic acids, and complex macromolecules such as humics (reviewed by Bronk 2002).

Traditional views of the marine N cycle held that phytoplankton used DIN while bacteria remineralized DON into the inorganic forms supporting primary production. Research over the past three decades, however, has shown not only that bacteria balance their organic N

consumption with uptake of inorganic nutrients (Wheeler & Kirchman 1986, reviewed by Kirchman 2000), but also that phytoplankton can use DON to meet cellular N demands (Palenik & Morel 1990, Antia et al. 1991, Berman et al. 1991, Ietswaart et al. 1994, Berman & Chava 1999, reviewed in Bronk 2002, and Berman & Bronk 2003). In fact, DON uptake has been shown in some studies to satisfy a large proportion of the N requirement in autotrophs. For example, the contribution of DON substrates such as urea, DFAA, and DCAA to total measured N uptake in coastal systems is variable, but has been shown to reach as high as 60% (Harrison et al. 1985, Glibert et al. 1991). This should not be surprising, since DON typically comprises the majority of the total dissolved N (TDN) pool, roughly 60-70% on average in coastal and oceanic surface waters (Bronk 2002). Studies of autotrophic DON use have been limited, however, by the availability of ^{15}N-labeled organic substrates and also by the fact that most of the DON pool has not been characterized. To date, most uptake studies have used ^{15}N-labeled urea and amino acids to examine DON utilization because they are important to microbial N nutrition and are commercially available.

The uptake of both inorganic and organic N by various constituents within the microbial community was measured during the Geochemical Rate/RNA Integration Study (GRIST), a coordinated field experiment exploring the correlation between biogeochemical flux rates, gene expression, and mRNA abundance to demonstrate the application of molecular approaches to studying marine carbon (C) and N cycles. Conducted in continental shelf waters off the southern coast of New Jersey from 19-25 July 2002, the GRIST project studied the variation in microbial activity over two diel periods (Kerkhof et al. 2003). In this component of GRIST, we characterized ambient nutrient concentrations and measured uptake rates of DIN (NH_4^+, NO_3^-, and NO_2^-) and DON (urea and DFAA) in various size fractions during both diel studies. Using

sequence analysis of the *ure*C genes present in surface waters, we also determined the bacterial and picoeukaryotic taxa capable of utilizing urea. To date, little is known about the phylogenetic diversity of genes responsible for urea assimilation in coastal zones, especially through the use of cultivation-independent approaches.

MATERIALS AND METHODS

Study site and field sampling

The GRIST experiment was conducted at a Long-Term Ecosystem Observatory site (LEO-15) established by the Mid-Atlantic Bight (MAB) National Undersea Research Center in 1996 (Glenn et al. 1996). LEO-15 is located in 15 m of water on the inner shelf directly offshore from the Rutgers University Marine Field Station (RUMFS) in Tuckerton, New Jersey. Using RUMFS as a base, GRIST consisted of two diel experiments, hereafter referred to as Diel 1 (20-21 July 2002) and Diel 2 (22-23 July 2002). Sampling occurred at roughly four-hour intervals, whereupon water was collected from the surface (~1 m) and bottom (~14 m) of the water column. Samples for molecular analyses were filtered and flash frozen on station aboard the R/V *Arabella*. Water for nutrient analyses and ^{15}N uptake experiments was transferred using a pump and hose set-up to 20 L acid-washed plastic carboys, shaded with neutral density screening, and transported to the RUMFS laboratory within 45 minutes of collection.

Nutrient analyses

At each time point, water from both depths was filtered through combusted Whatman GF/F filters, frozen, and analyzed later in the laboratory to determine nutrient concentrations. Filtered water samples for the determination of NH_4^+ concentrations were not frozen, but rather refrigerated upon addition of the phenol-alcohol reagent, which binds available NH_4^+, and analyzed at RUMFS within 24 h of sampling using the manual phenol-hypochlorite method (Parsons et al. 1984). An AlpKem AutoAnalyzer was used to measure NO_3^- and NO_2^- concentrations, urea was measured using the manual monoxime method (Price & Harrison 1987), and DFAA concentrations were analyzed as the individual compounds by high-performance liquid chromatography using *o*-phthaldialdehyde (Lindroth & Mopper 1979). Concentrations of DON were determined as the difference between TDN and DIN; TDN was measured with the persulfate oxidation technique (Bronk et al. 2000).

Uptake experiments

Stable isotope tracer techniques were used to quantify uptake rates of inorganic and organic N by various size fractions within the microbial community. To this end, the following five substrates were added separately to collected water samples: ^{15}N-labeled NH_4^+, NO_3^-, and NO_2^-, and dual-labeled (^{15}N, ^{13}C) urea and DFAA (an algal extract consisting of sixteen amino acids; Cambridge Isotope Laboratories, Andover, MA). Furthermore, multiple incubations were conducted to evaluate the uptake attributed to the following size fractions: >5.0 μm (Diel 1 only), >3.0 μm (Diel 2 only), GF/F (0.7 μm nominal pore size), >0.8 μm (NH_4^+ and NO_3^- only),

0.2-0.8 μm (NH_4^+ and NO_3^- only), and >0.2 μm. When possible, data on ambient nutrient concentrations were obtained from previous studies to estimate tracer additions that would yield an enrichment of less than 10% over the ambient concentrations; the initial and final substrate isotopic enrichments were later calculated as in Bronk et al. (1998).

At each time point, uptake experiments were performed in 500 ml PETG bottles, with separate incubations for each size fraction, depth, and substrate. After addition of labeled substrates, the bottles were incubated outside for approximately one hour in flow-through coolers kept at *in situ* light and temperature conditions. Incubations were terminated by filtration through either glass fiber filters (GF/F) or silver membrane filters (5 μm, 3 μm, 0.8 μm, 0.2 μm). In addition to the standard three filters used for every substrate (5 μm or 3 μm, GF/F, 0.2 μm), samples spiked with $^{15}NH_4^+$ or $^{15}NO_3^-$ were also divided into the >0.8 μm and the 0.2-0.8 μm fractions to examine the role of the bacterial size fraction in uptake of these inorganic forms. All filters used to terminate the incubations were frozen and returned to the laboratory for analysis on a Europa GEO 20/20 dual isotope mass spectrometer, providing both particulate N (PN) concentrations as well as isotopic atom percent enrichments in the PN pool for each substrate and size fraction. Nitrogen uptake rates were calculated as described in Bronk et al. (1998). Furthermore, the NH_4^+ pool was isolated using solid phase extraction (Dudek et al. 1986, Brzezinski 1987), and NH_4^+ uptake rates were corrected for isotope dilution from N regenerated during the course of the incubation, as described in Glibert et al. (1982).

Amplification, cloning and sequencing of *ure*C

Genomic DNA was extracted from cells collected in the 0.2-0.8 μm and 0.8-3.0 μm size fractions by filtration. Thirty liters of seawater was filtered through a 3.0 μm Versapor pleated capsule (Pall, East Hills, NY) and then sequentially passed through 142 mm, 0.8 μm and 0.2 μm Supor membrane filters. The 0.2 and 0.8 μm filters were immediately frozen and later pulverized before extracting genomic DNA using the UltraClean mega soil DNA kit (MoBio, Carlsbad, CA).

Amplification of *ure*C genes took place in 25 μl reactions containing the following components: 12.5 μl Qiagen HotStar master mix, 0.5 μM of each primer and 10 ng of genomic DNA. The forward primer (*ure*CnineF: 5' GARGTIAAITTYGGIGGIGGIAARGT 3', where I = inosine, R = A or G, and Y = C or T; translates to EVKFGGGKV and corresponds to nucleotide positions 127-152 of the *E. coli* 0157:H7 *ure*C [AAG55290]) was paired with one of the following reverse primers: *ure*CfiveRev (5'TCRTGIAGIAYRTCYTCIGCIGCIAT 3'; translates to IAAEDVLHD and corresponds to nucleotide positions of 1027-1052 of the *E. coli* 0157:H7 *ure*C) or *ure*CsixRev (5' ATRTCYTCIGCIGCIATIGTYTC 3'; translates to ETIAAEDV and corresponds to nucleotide positions 1021-1043 of the *E. coli* 0157:H7 *ure*C), to form products of approximately 926 and 917 bp, respectively. The *ure*C gene of *Silicibacter pomeroyi* (Moran et al. 2004) was successfully amplified with either primer set under these conditions. Priming sites were chosen to maximize inclusiveness and amplify a large portion of the gene. Based on inspection of *ure*C gene and amino acid alignments, the primers designed here target nearly all available sequences of most gram negative bacteria and eukaryotic algae. Since the *ure*C sequences of eukaryotic algae available in GenBank at this time

(*Pseudoisochrysis paradoxa* [AF432601], *Tetraselmis* sp. CCMP1613 [AF432600], *Rhodomonas salina* [AF432599], *Phaeodactylum tricornutum* [AF432598], and *Chlamydomonas* sp. CCMP 222 [AF432597]) are partial in length, it is unknown whether they are compatible with the forward primer. However, at least one of the reverse primers matches the *ure*C genes of each species. The PCR products generated using a 52°C annealing temperature and 35 cycles were agarose gel-extracted using a Freeze N' Squeeze™ spin column (Bio-Rad Laboratories, Hercules, CA.) and subsequently cloned using the TOPO TA cloning vector for sequencing kit (Invitrogen, Carlsbad, CA). Four total libraries were constructed; one library was generated from each DNA sample (0.2-0.8 μm and 0.8-3.0 μm fractions of a sample taken at 1 m depth on 18 July 2002 at 20:00) using both primer sets (*ure*CnineF/*ure*CfiveRev and *ure*CnineF/*ure*CsixRev). Fifteen clones from each library were extracted, sequenced and phylogenetically analyzed as described elsewhere (Allen et al. 2002). These *ure*C sequences were deposited in GenBank and are represented by the accession numbers DQ286064 through DQ286116. GenBank sequences that partially overlapped with our amplicon were analyzed but not included in the phylogenetic dendrogram since this would have required utilizing significantly less sequence information (~1/3) than was generated.

RESULTS

Environmental conditions

The MAB region around the LEO-15 study site is often subjected to strong alongshore winds from the south that drive episodic upwelling typically lasting from days to weeks (Glenn

et al. 1996). This upwelling entrains nutrient-rich bottom water from offshore into the surface layer, thus stimulating phytoplankton blooms and organic matter accumulation (Hicks & Miller 1980, Clemente-Colón 2001, Vlahos et al. 2002). Temperature and fluorescence profiles obtained by the LEO-15 monitoring node suggest that an upwelling event occurred around 10-12 July, with a possible smaller mixing event from 18-20 July. However, there was an increase in stratification at the start of Diel 1 and the water column was well-stratified through Diel 2, with a thermocline between 6 and 8 m depth (see Fig. 1 in Corredor et al. 2004). Surface water temperatures increased from 19°C to 22°C during Diel 1 then ranged between 22°C and 24°C during Diel 2 as stratification strength increased. Bottom water temperatures were from 15-17°C and 16-18°C for Diels 1 and 2, respectively. Salinity remained relatively constant during Diel 1, increasing slightly from 31.6 at the surface to 32.0 in the bottom water, and fluctuated very little through Diel 2. Chlorophyll measurements, corroborated by fluorometry data from LEO-15 node A, indicated a small but distinct phytoplankton bloom that appeared to intensify during Diel 1, and peaked late in Diel 2 (see Fig. 2 in Corredor et al. 2004). Particulate N data support this claim (Fig. 1), with PN concentrations in the surface layer increasing from the start of Diel 1, with a small peak at 16:00, to about 12:00 during Diel 2, at which point the PN levels decline. The bottom-water PN concentrations varied less than those in the surface, and were roughly equivalent between Diel 1 and Diel 2 (Fig. 1); these bottom-water PN values agree well with the trends in chlorophyll data presented in Corredor et al. (2004). Overall, the 0.2-0.8 μm size fraction had the lowest PN, followed by the larger phytoplankton fraction (>5 or >3 μm) and the >0.8 μm size fraction, and the >GF/F fraction had the highest PN concentrations (Fig. 1).

Nutrient concentrations

Nutrient concentrations were similar across both diel periods, but were distinct between the surface and bottom layers of the water column (Fig. 2). The surface layer was virtually devoid of DIN; DON comprised 99-100% of the TDN concentrations, which averaged 7.4 μM N and 8.7 μM N for Diel 1 and 2, respectively (data not shown). Urea was the most abundant form of N measured in the ambient surface pool (26% of the TDN concentration), followed by DFAA (3%). Other, unidentified forms of DON represented the remaining 70-71% of the TDN pool (Fig. 3). All NH_4^+ concentrations were at or below detection (0.05 μM N) in the surface layer. Similarly, combined NO_3^- and NO_2^- concentrations (NO_X^-) were below detection (0.03 μM) for nearly all surface samples (Fig. 2).

The characteristics of the ambient TDN pool in the bottom water at LEO-15 were entirely different from that of the surface layer. Roughly divided into thirds between NH_4^+, NO_X^-, and DON, the mean TDN concentration was 12.6 μM N for both Diel 1 and 2 despite their difference in respective ranges (12.1-12.8 μM N vs. 10.2-14.0 μM N). In sharp contrast with the surface, DIN accounted for up to 67% of the ambient TDN (mean of 59%) in the bottom water, whereas urea and DFAA comprised much less of the total than in the surface despite concentrations that were only slightly lower. Urea ranged from 13 to 18% over both diels, while DFAA contributed only 1% to TDN on average. The DON pool as a whole represented 33 to 40% (mean of 38%) of bottom-water TDN during Diel 1 and from 35 to 54% (mean of 44%) during Diel 2 (Fig. 3).

Uptake experiments

Reflective of nutrient availability, N utilization by the microbial community was also distinct between the surface and bottom waters at LEO-15 (Fig. 4). Uptake by cells retained on a GF/F filter was dominated by urea at the surface (≤ 77% of total uptake), while NH_4^+ contributed most to total measured uptake in the bottom waters (≤ 66%; Fig. 3). Note that Fig. 3 presents the means of the two diels, whereas percentages given in the text are ranges from both diel experiments. On average, absolute uptake rates (ρ, μM N h^{-1}) for all substrates were greater during Diel 2 than Diel 1 (Fig. 4), which is consistent with a developing phytoplankton bloom over the course of the experiment.

Surface. In the surface water, absolute urea uptake rates were about three times those of NH_4^+ and at least an order of magnitude greater than those of the other three substrates (Fig. 4). There was no significant change in mean urea uptake rate between diel experiments ($p = 0.351$), nor was the difference significant for urea's relative contribution to total uptake ($p = 0.104$). Absolute uptake rates for DFAA accounted for 3-8% of the total uptake, and together these two DON forms represented from 51 to 79% of the total measured N uptake in the surface water. However, DIN uptake rates indicate that inorganic N forms were absent from the ambient surface pool, likely a result of their rapid turnover by phytoplankton and bacteria. Ammonium uptake rates (range of 0.308-0.580 μM N h^{-1}), when corrected for isotope dilution, contributed 17-41% of the total measured N uptake, with NO_X^- (0.072-0.214 μM N h^{-1}) comprising another 4-11%; overall, DIN represented 30% of the total measured uptake. Finally, there were no clear diel patterns observed in surface water N uptake for any of the five substrates studied.

Bottom. Nitrogen uptake dynamics in the bottom water were quite different from those in the surface layer. Dissolved inorganic N was the major source of N to the microbial community in the bottom water and accounted for up to 85% of the total uptake on GF/F filters, with a mean of 72% across both diel periods (Fig. 3). Absolute NH_4^+ uptake rates, which exceeded those of all other substrates, decreased over the course of each diel experiment from an early morning maximum (Fig. 2), but also appeared to increase from Diel 1 (0.058 μM N h^{-1}) to Diel 2 (0.095 μM N h^{-1}), although the difference was not significant at the 95% level ($p = 0.064$). The second highest rates in the bottom water were those of NO_X^-, and while NO_X^- uptake rates did not show a distinct temporal trend, the relative contribution of NO_X^- to total uptake tended to increase with time of day (Fig. 3). Although all three DIN species represented a greater proportion of the total uptake in the bottom water, their respective uptake rates were about four to six times higher in the surface water. Dissolved organic N, on the other hand, contributed much less to the total uptake in the bottom water compared to the surface, with urea and DFAA together representing 33% of the total uptake in Diel 1 and 26% during Diel 2. Mean uptake rates of these two DON forms were roughly similar across both diel experiments. In general, absolute N uptake rates for all substrates were higher during Diel 2 than Diel 1.

Size-fractionated uptake

Uptake rates for all five N substrates were measured in three different size fractions: >GF/F (~0.7 μm), >5 μm (Diel 1) or >3 μm (Diel 2), and >0.2 μm; additionally, uptake of NH_4^+ and NO_3^- were measured in the >0.8 μm (phytoplankton) and 0.2-0.8 μm (bacteria) size fractions. Size-fractionated NO_2^- uptake rates were determined during Diel 1 only. Nitrogen-

specific uptake rates (ν, h^{-1}) are used throughout this section in place of absolute rates to compare the physiological N metabolism of cells from distinct size fractions within the microbial community. Surface-water specific uptake rates were typically greatest in the >GF/F fraction and lowest in the >0.2 μm fraction (Figs. 5-8), presumably as a result of decreased ^{15}N enrichment in the bacterial fraction. In the bottom water, however, specific rates were often highest in the >0.2 μm size fraction and more so with DIN than DON substrates, which lends support to the argument that bacterial uptake significantly affected the total uptake in this size fraction. Specific uptake rates measured during Diel 2 generally exceeded those of Diel 1 in all size fractions, and these rates were generally higher in surface waters.

Results indicate that although uptake rates differed greatly between size fractions and substrates, the relative contribution of each substrate to the total measured uptake was similar across size fractions, with a few notable exceptions described below. In the surface layer, specific N uptake in all three size fractions (>0.2 μm, >GF/F, and >3 or 5 μm) was dominated by urea, and the relative contribution of this organic substrate to the total measured uptake increased with cell size. For example, on average urea comprised 61%, 68%, and 75% of the total uptake in the >0.2 μm, >GF/F, and >3 or 5 μm size fractions, respectively. Although the relative contribution of urea to total uptake in the surface was greatest in the larger phytoplankton fraction, specific urea uptake rates were highest in the >GF/F fraction (0.084 h^{-1}) and lowest in the >0.2 μm fraction (0.007 h^{-1}; Fig. 7). The other form of organic N used in this study was DFAA, which represented just 2-4% of the total uptake in the >3 or 5 μm size fraction and 3-7% in the >GF/F fraction with relatively low uptake rates (Fig. 8); however, this relative contribution increased to 6-38% (mean of 17%) in the >0.2 μm fraction as more bacterial uptake was included.

Ammonium was the preferred form of inorganic N taken up by cells from all size fractions in the surface layer, with a percent relative uptake of 17%, 16%, and 20% for the >0.2 μm, >GF/F, and >3 or 5 μm size fractions, respectively (Fig. 5). Uptake rates for NO_3^- and NO_2^-, on the other hand, were relatively low (Fig. 6; NO_2^- data not shown) and together represented up to 11% of the total uptake when NO_2^- uptake was measured, but averaged 6-7% for all three size fractions.

Nitrogen use across the three main size fractions, like the ambient nutrient regime, was nearly reversed in the bottom water compared to the surface. Inorganic N, and particularly NH_4^+, was the dominant form taken up by all size fractions in the bottom samples, with DIN representing 81%, 74%, and 68% of the total measured uptake in the >0.2 μm, >GF/F, and >3 or 5 μm size fractions, respectively. The relative contribution of DON (urea and DFAA) uptake increased with cell size, from 19% in the >0.2 μm fraction to 26% on GF/F filters to 32% in the larger phytoplankton fraction. In all size fractions, NH_4^+ uptake rates exceeded those of the other substrates (Figs. 5-8). In most cases, NO_3^- had the second-highest uptake rates, which represented 29% of the total uptake in the >0.2 μm fraction, decreasing to 15% and 12% in the >GF/F and >3 or 5 μm fractions, respectively. When measured, NO_2^- uptake rates were often higher than those of NO_3^- and contributed 11-31% of the total uptake in the >0.2 μm fraction, and less than 11% in the other two size fractions. Uptake rates of urea and DFAA (Figs. 7-8) occasionally equaled or exceeded those of NO_3^-, but on average contributed less to total uptake.

Finally, N uptake was examined in the >0.8 μm and 0.2-0.8 μm size fractions in order to investigate how and when the bacterial community might utilize DIN substrates to supplement cellular N demand. In most cases, NH_4^+ uptake rates were greater than those of NO_3^- in both the phytoplankton (>0.8 μm) and the bacterial (0.2-0.8 μm) size fractions (Figs. 5-6). The bacterial

size fraction was responsible for 41% and 34% of the surface NH_4^+ uptake during Diel 1 and Diel 2, respectively, and 61% and 52% of the corresponding NH_4^+ uptake in the bottom water. For NO_3^-, this bacterial contribution was 34% and 26% of the total at the surface during Diel 1 and 2, respectively, and 73% and 39% in the bottom water. Thus, heterotrophic bacteria represented a significant component of the DIN uptake throughout the water column, and were often responsible for at least half the NH_4^+ and NO_3^- uptake in the bottom water during the two diel experiments.

*ure*C diversity

In order to determine the diversity of microbes capable of utilizing urea in the <3.0 μm size class, we designed and applied PCR primers targeting the gene (*ure*C) that encodes for the large catalytic α subunit of the urease enzyme (Mobley et al. 1995). A total of 53 sequences derived from four clone libraries were recovered from a surface sample; each recovered sequence was distinct from those present in GenBank (Fig. 9). The GenBank-derived *ure*C sequences that were theoretically amplifiable with our primer sets fell into 10 clades (arbitrarily referred to as 1-10 in Fig. 9), six of which contained LEO-15 sequences and four of which contained sequences recovered from the Sargasso Sea metagenomic library (Venter et al. 2004). Similar to the Sargasso Sea *ure*C genes, the majority of the LEO-15 sequences were affiliated with those of the *Cyanobacteria* (47%) and the alpha *Proteobacteria* (30%). The *Cyanobacteria* clade consisted of *ure*C sequences from eleven cultivated species. Based on a criterion of 98% amino acid identity, two types of *Cyanobacteria*-like sequences were recovered among the LEO-15 clones: one group consisted of 23 highly similar sequences (sharing 98-100% amino acid

identity) that were approximately 95% identical at the amino acid level to two highly similar LEO-15 sequences (sharing 98% amino acid identity). Both groups were most similar (95-96% amino acid identity) to the *ure*C genes of *Synechococcus* sp. WH7805 and WH8102 and to two Sargasso Sea clones (EAI52258 and EAJ32162). A comparatively higher diversity of alpha *Proteobacteria*-like *ure*C sequences was recovered. Sixteen LEO-15 clones associated with this group share an amino acid identity ranging between 80-100% and are composed of seven distinct sequence types based on a 98% amino acid identity cut-off. These sequences were most closely related to those of the bacteria *Silicibacter pomeroyi* and *Silicibacter* sp. TM1040 (up to 93.5% amino acid identity) and several Sargasso Sea clones (up to 96.4% amino acid identity). The organisms corresponding to the *ure*C sequences in this clade are all members of the alpha *Proteobacteria* subphylum. The remaining LEO-15 sequences (23%) were affiliated with four distinct clades and did not share high sequence identity with any *ure*C sequence present in GenBank (77 - 84% amino acid identity). As a result, the phylogenetic group of the corresponding organisms cannot be inferred.

The largest proportion of LEO-15 sequences recovered from the 0.2-0.8 μm size class were members of the alpha *Proteobacteria* (46%) cluster, while the sequences in the 0.8-3.0 μm fraction consisted primarily of *Cyanobacteria* (70%), regardless of the primer pair used. There was extensive redundancy in the types of *ure*C sequences recovered from each size fraction. The vast majority (92.5%) of 0.8-3.0 μm sequences shared at least 98% amino acid identity with at least one sequence isolated from the 0.2-0.8 μm libraries. Unfortunately, no molecular data from cells retained by the 3 μm filter exists to complement the uptake data for the >3.0 μm fraction.

DISCUSSION

Uptake of inorganic and organic nitrogen forms

The results presented here feature an ecosystem with vastly different N uptake regimes in the surface and bottom layers of a stratified water column. Organic N (urea) clearly dominated N uptake by the plankton community in the surface layer, while DIN (primarily NH_4^+) fueled most of the microbial N uptake in the bottom water. Furthermore, the relative contribution of urea to total uptake in the surface water (up to 77%), along with the magnitude of the uptake rates and ambient urea concentrations, are quite high relative to other marine ecosystems studied (reviewed by Bronk 2002). For example, Lomas et al. (2002) compiled data on urea concentrations and uptake rates measured along Chesapeake Bay between 1972 and 1998 and reported that concentrations rarely exceeded 1.5 μM N, with the average annual concentration ranging between 0.49 and 0.91 μM N. Urea concentrations measured at LEO-15 during this study were between 1.4 and 2.7 μM N. The maximum absolute urea uptake rates cited by Lomas et al. (2002) over the 26-year period were almost 1 μM N h^{-1} (in 1997); in this study, the maximum measured uptake was 1.84 μM N h^{-1}. Furthermore, the highest reported contribution, to the authors' knowledge, of urea to total uptake in a marine environment was 60-80%, measured in the Chesapeake Bay plume during the winter and summer of 1985 (Glibert et al. 1991). The corresponding contribution from GF/F filters reported here was 47-77% in the surface water. The urea uptake rates and concentrations measured in the surface layer at LEO-15 were also considerably higher than previously measured values in a number of other ecosystems

(Bronk 2002). However, a recent study in the Neuse River Estuary, North Carolina, measured urea uptake rates as high as 3.72 μM h^{-1} (Twomey et al. 2005).

Ambient concentrations and uptake rates for the other substrates studied in this experiment are more consistent with previously reported data from various marine ecosystems, if not specifically characteristic of the inner continental shelf. For example, the absence of a standing stock of DIN in the surface water resembles an oligotrophic oceanic gyre, yet the surface uptake rates are comparable to results from some coastal and estuarine systems (Bronk et al. 1998, Bronk & Ward 1999, Berg et al. 2001, Veuger et al. 2004). Concentrations and absolute uptake rates for DFAA at LEO-15 were between 0.09-0.69 μM N and 0.011-0.179 μM N h^{-1}, respectively, which are generally within, but at the upper end, of values reported elsewhere (Bronk 2002). The DFAA uptake rates reported here are similar to the range measured along the Thames estuary (0.006-0.15 μM N h^{-1}) by Middelburg and Nieuwenhuize (2000).

Due to the low ambient concentrations of NH_4^+, NO_3^-, and NO_2^- in the surface water, the 0.1-0.2 μM addition of each substrate, albeit very small, still represented 65-100% of the ambient pool at the start of the incubations. As a result, the measured uptake rates for these substrates were likely enhanced in the surface water, which suggests that the relative importance of urea uptake *in situ* was even greater than our uptake rates indicate. During the incubations, however, the stimulating effect of the $^{15}NH_4^+$ tracer addition was minimized due to relatively high NH_4^+ regeneration rates that exceeded 1.0 μM h^{-1} in about half of all samples, with a maximum rate of 3.0 μM h^{-1} (data not shown). Furthermore, such additions of DIN tracer essentially mimic the nutrient pulses concurrent with the episodic upwelling events that are common to this study area.

In the bottom water, however, additions of $^{15}NH_4^+$, $^{15}NO_3^-$, and $^{15}NO_2^-$ represented 2-8%, 4-13%, and 7-25% of the ambient pool, respectively. Urea additions to both surface and bottom samples were also kept close to the 10% enrichment target, ranging from 4 to 12% of the ambient pool. DFAA additions were as low as 15% for one sample, but were approximately equal to ambient concentrations for most samples, and as high as 213% of ambient DFAA in the extreme. Thus, high enrichment (> 10%) of the available pool may have artificially enhanced uptake rates of DFAA.

Substrate preferences within the plankton community

Results from size-fractionated uptake experiments can be used to assess patterns of N preference for various components of the microbial community in both the surface and bottom mixed layers. For example, in the surface layer urea dominated N uptake in the >GF/F size fraction with a mean contribution of 68% of the total measured uptake, which increased to 75% in the larger size fraction (>3 and 5 μm), but decreased to 61% when the bacterial community was included on 0.2 μm filters. This suggests that most of the urea uptake at LEO-15 was by the larger phytoplankton community, and that bacteria in general did not prefer urea as a source of N. The bacterial community in the surface water did, however, appear to prefer DFAA as a source of N and C; the relative contribution of this substrate to total uptake was 5% in the GF/F fraction, 3% in the >3 or 5 μm fraction, but 17% in the >0.2 μm size fraction. Nonetheless, the finding that DFAA uptake represented a mean of 6% in both surface and bottom waters and up to 16% of the total uptake by the larger phytoplankton community is significant because amino acids have traditionally been neglected as a source of N to this group, despite evidence that

DFAA are utilized directly in varying amounts by autotrophs, and even indirectly via extracellular enzymatic hydrolysis of peptides and proteins (Palenik & Morel 1990, Mulholland et al. 2002, Mulholland et al. 2003, Stoecker & Gustafson 2003).

The bacterial community in the bottom water at LEO-15 appeared to rely less on DFAA and urea to meet their N demand, and more on inorganic N sources. This is suggested by the increase in the relative contribution of DIN substrates to total uptake as more bacteria were included, from 68% in the >3 or 5 μm fraction to 74% on GF/F filters and 81% in the >0.2 μm size fraction. Ammonium contributed the most to total uptake in the bottom water for all size fractions, but contributed slightly less in the >0.2 μm size range relative to the larger two fractions. Nitrate, on the other hand, played a greater role when the bacterial community was included, with the relative contribution nearly doubling between the >GF/F and >0.2 μm fractions. Thus, bacteria appeared to rely on NO_3^- (and NO_2^-) to supplement their cellular N demand in bottom waters.

Results from the size fractionation experiments using 0.8 and 0.2 μm silver filters with NH_4^+ and NO_3^- additions were in agreement with the conclusion that the bacterial community relied on DIN to supplement organic N use. For example, bacterial uptake (0.2-0.8 μm fraction) was 21-49% of the total NH_4^+ uptake in surface water and 41-72% in bottom water. Bacterial NO_3^- use ranged from 21-43% of the total in the surface and 32-93% in the bottom layer. By combining these results with those from the main size fractionation experiments, it is apparent that bacteria were responsible for a substantial portion of the DIN use in both surface and bottom waters and were not simply remineralizing organic matter, but also competing effectively with phytoplankton for available inorganic N, particularly NO_3^-. Despite the dogma that marine bacteria are not significant consumers of NO_3^-, various researchers have indeed shown, as is the

case here, that NO_3^- can support growth of heterotrophic bacteria in multiple marine ecosystems (Kirchman et al. 1991, Kroer et al. 1994, Kirchman & Wheeler 1998, Allen et al. 2002, Allen et al. 2005). Enhanced bacterial dependence on DIN in continental shelf waters may even exert a selective pressure that favors phytoplankton cells capable of either competing effectively with bacteria for available DIN, or using available DON, such as urea.

Organisms capable of urea utilization in LEO-15 surface waters

The *ure*C sequences recovered from the LEO-15 surface waters were diverse and represent microbes whose *ure*C genes have not been deposited in GenBank thus far. The high proportion of 0.8-3.0 μm-derived sequences having > 98% amino acid identity to those of the bacterial fraction implies that the majority of *ure*C genes retrieved in this study were bacterial. Based on our phylogenetic analysis, the *Cyanobacteria* and members of the alpha *Proteobacteria* appear to represent two major groups capable of urea assimilation in the surface waters of the LEO-15 site. It is not surprising that the *ure*C genes recovered displayed such affiliation considering the ubiquitous distribution and numerical dominance of these groups in marine systems (Giovannoni & Rappé 2000). For instance, the SAR11 group, a constituent of the alpha *Proteobacteria*, is thought to be the most dominant group of bacterioplankton in the sea (Rappé et al. 2002). Another alpha proteobacterial group, the marine *Roseobacter* (of which *Silicibacter* is a member), is estimated to represent 10-20% of the 16S rRNA genes recovered from oceanic surface waters (Giovannoni & Rappé 2000). Furthermore, it has been established that many marine cyanobacteria, including *Synechococcus* spp., possess *ure*C genes and can utilize urea as a sole N source (Collier et al. 1999, Moore et al. 2002). The fact that both groups represent the

majority of *ure*C sequences in the LEO-15 libraries and the Sargasso Sea metagenomic database suggests that they may be significant constituents of the urea-assimilating community in marine systems. These sample sets represent systems distinct in nature (relatively nutrient-rich, turbid, shallow coastal waters versus nutrient-deficient, clear open ocean) and the *ure*C genes were collected by very different means (direct cloning versus primer-based amplification of genomic DNA). While this study demonstrates that these organisms were present and potentially capable of utilizing urea at the LEO-15 site, the data provided here suggests that they did not contribute significantly to total urea uptake. Since diatoms and chlorophytes were largely responsible for carbon fixation in surface waters during this experiment (Corredor et al. 2004), members of these groups may have been responsible for the observed urea utilization and were simply not recovered here because the >3.0 μm size class was not processed for *ure*C analysis. High urea availability coupled with a bacterial community that appears to be meeting its N demand through alternative sources such as DFAA may explain the successful acquisition of urea by larger phytoplankton in the LEO-15 surface waters.

Ecosystem dynamics and sources of nitrogen at LEO-15

Exchange of nutrients between surface and bottom waters was restricted by a significant pycnocline at 6-8 m depth over the course of both diel experiments (Corredor et al. 2004). Comparisons of nutrient concentrations in both mixed layers indicated that TDN concentrations were higher in the bottom water (12.6 μM) than at the surface (8.0 μM; $p < 0.00001$) and increased with time at the surface but not at the bottom. Concentrations of DON, however, were higher at the surface (8.0 μM) than at the bottom (5.1 μM; $p < 0.001$), and increased slightly

from Diel 1 to Diel 2 at both depths. One hypothesis explaining the ecosystem N dynamics observed during these two diel experiments at LEO-15 is that a small phytoplankton bloom developed, as evidenced by increasing chlorophyll and PN concentrations from Diel 1 to Diel 2. This small bloom had depleted the standing stocks of DIN and caused an accumulation of DOM in the surface layer, thereby increasing ambient DON concentrations. A concurrent high mineralization rate in the bottom waters, due to the increased sedimentation of particulate organic matter (POM) from the surface bloom, could have caused the decreased abundance of DON in bottom waters coupled with persistently elevated NH_4^+ concentrations. The changes in relative contribution of NO_X^- and DFAA to total uptake when bacteria were included in size fractionation experiments suggests that bacterial activity was high relative to other plankton groups. Increased activity by heterotrophic bacteria, especially in the sediments, as well as low dissolved oxygen and higher concentrations of NH_4^+, are all typical of coastal environments as a result of enhanced delivery of exported POM to the sediments (Capone 2000). Benthic release of inorganic nutrients after periods of increased primary production can subsequently be a significant source of N to phytoplankton throughout the water column (Nixon & Pilson 1983, Boynton et al. 1995).

Finally, there is additional evidence suggesting that NH_4^+ remineralized in the bottom water and sediments is subsequently nitrified to NO_X^-, as seen in the high concentrations of the intermediate product NO_2^- relative to the ambient NO_3^- concentrations (Fig. 2). Furthermore, specific uptake rates for NO_2^- measured in the bottom water represented 3-11% of the total uptake in the >GF/F and >3 or 5 μm size fractions, but increased to 11-31% when the bacterial community was included on 0.2 μm filters. This suggests that nitrifying bacteria may have been particularly abundant in the bottom water during this study. In a study of N removal by

denitrification in LEO-15 sediments, Laursen and Seitzinger (2002) saw evidence of pelagic nitrification during summer stratification and also found that nitrification rates in sediments were, on average, slightly higher than those for denitrification. Coupled nitrification-denitrification is an important process in the N cycle of coastal ecosystems, and likely played a role at this study site, with pelagic and benthic nitrification providing the substrate required for denitrification in sediments low in oxygen due to organic matter mineralization (Capone 2000). Therefore, high concentrations of NO_X^- measured in the bottom layer at LEO-15 may have been due mostly to nitrification rather than allochthonous sources such as advection or riverine input.

Certainly other scenarios are possible to explain the source of DIN to the bottom waters. For example, the DIN concentrations measured in the bottom water may have been supplied by an upwelling event bringing nutrient rich water from the outer shelf prior to the onset of stratification, after which the surface DIN pool was depleted by microbial uptake as described above. Since temperature profiles from the LEO-15 monitoring nodes do not show any evidence of such upwelling during the course of these experiments, this allochthonous DIN supply would have had to originate from an earlier event. Nonetheless, a stratified water mass with nutrient-rich bottom water could have advected into the study area, and this could potentially explain the decrease in DON concentrations and increased NO_2^- abundance measured between diels.

This research has demonstrated the complexity that often exists in coastal ecosystems with respect to the availability, uptake, and preference of inorganic and organic N compounds by the microbial community. The results have also shown that heterotrophic bacteria can effectively compete with phytoplankton for DIN in a N-limited environment, potentially forcing the phytoplankton to rely on available forms of organic N, such as urea. Additionally, this represents, to the authors' knowledge, the first published study of *ure*C gene diversity in coastal

upwelling waters and is a first step in the characterization of marine populations involved in urea utilization.

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Figure Captions

Fig. 1. Particulate nitrogen (PN) concentrations measured in surface and bottom waters at LEO-15 during two diel experiments: Diel 1, 20-21 July 2002 and Diel 2, 22-23 July 2002. Note the five-fold decrease in scale between surface and bottom. Error bars denote ± 1 SD of the mean. Shaded bars indicate dark periods. Data from Diel 1 Bottom, time point five, does not appear here or in any other figures due to a sampling error.

Fig. 2. Dissolved nutrient concentrations measured in surface and bottom waters at LEO-15 during two diel experiments: Diel 1, 20-21 July 2002 and Diel 2, 22-23 July 2002. Note the two-fold increase in scale between surface and bottom. Error bars denote ± 1 SD of the mean. Shaded bars indicate dark periods. Surface concentrations of NH_4^+, NO_3^-, and NO_2^- were typically below detection (0.05, 0.03, 0.03 μM, respectively) and thus are not distinguishable from zero.

Fig. 3. Percentage of the total measured nitrogen concentrations and total measured GF/F uptake by substrate in surface and bottom waters at LEO-15. Data represents the mean contribution from both diel experiments.

Fig. 4. Absolute nitrogen uptake rates (ρ: μM N h^{-1}) measured using GF/F filters from two diel experiments at LEO-15 in July 2002. Note the ten-fold decrease in scale between surface and

bottom samples. Shaded bars indicate dark periods. The NH_4^+ uptake rate for Diel 1 Surface, first time point could not be corrected for isotope dilution and therefore is not shown here.

Fig. 5. Size-fractionated, specific NH_4^+ uptake rates (ν: h^{-1}) measured in surface and bottom waters at LEO-15 during two diel experiments: Diel 1, 20-21 July 2002 and Diel 2, 22-23 July 2002. Filled squares in Diel 2 represent the >3.0μm size fraction. Note the two-fold decrease in scale between surface and bottom. Shaded bars indicate dark periods.

Fig. 6. Size-fractionated, specific NO_3^- uptake rates (ν: h^{-1}) measured in surface and bottom waters at LEO-15 during two diel experiments: Diel 1, 20-21 July 2002 and Diel 2, 22-23 July 2002. Filled squares in Diel 2 represent the >3.0μm size fraction. Shaded bars indicate dark periods.

Fig. 7. Size-fractionated, specific urea uptake rates (ν: h^{-1}) measured in surface and bottom waters at LEO-15 during two diel experiments: Diel 1, 20-21 July 2002 and Diel 2, 22-23 July 2002. Note the twenty-fold decrease in scale between surface and bottom. Shaded bars indicate dark periods.

Fig. 8. Size-fractionated, specific DFAA uptake rates (ν: h^{-1}) measured in surface and bottom waters at LEO-15 during two diel experiments: Diel 1, 20-21 July 2002 and Diel 2, 22-23 July 2002. Shaded bars indicate dark periods.

Fig. 9. Dendrogram (~338 amino acids) displaying inferred phylogenetic relationships between LEO-15 clones and related *ure*C sequences recovered from GenBank (which are predominantly members of the *Proteobacteria*). Sequences recovered from the LEO-15 site are designated LEO and are surrounded by a box. Sequences recovered from libraries generated with primer pairs *ure*CnineF/*ure*CfiveRev or *ure*CnineF/*ure*CsixRev are designated as 95 (and an open circle) or 96 (and a filled circle), respectively. Sequences recovered from the 0.2-0.8µm or 0.8-3.0µm fraction end in .2 (open box) or .8 (shaded box), respectively. Identical sequences are listed adjacent to one another. Significant bootstrap values (>50%) are listed at the nodes of the tree. The *ure*C sequence from the fungal species *Schizosaccharomyces pombe* was used as the outgroup. GenBank accession numbers for the LEO clones are DQ286064 – DQ286116X; accession numbers for the remaining *ure*C sequences are shown in parentheses.

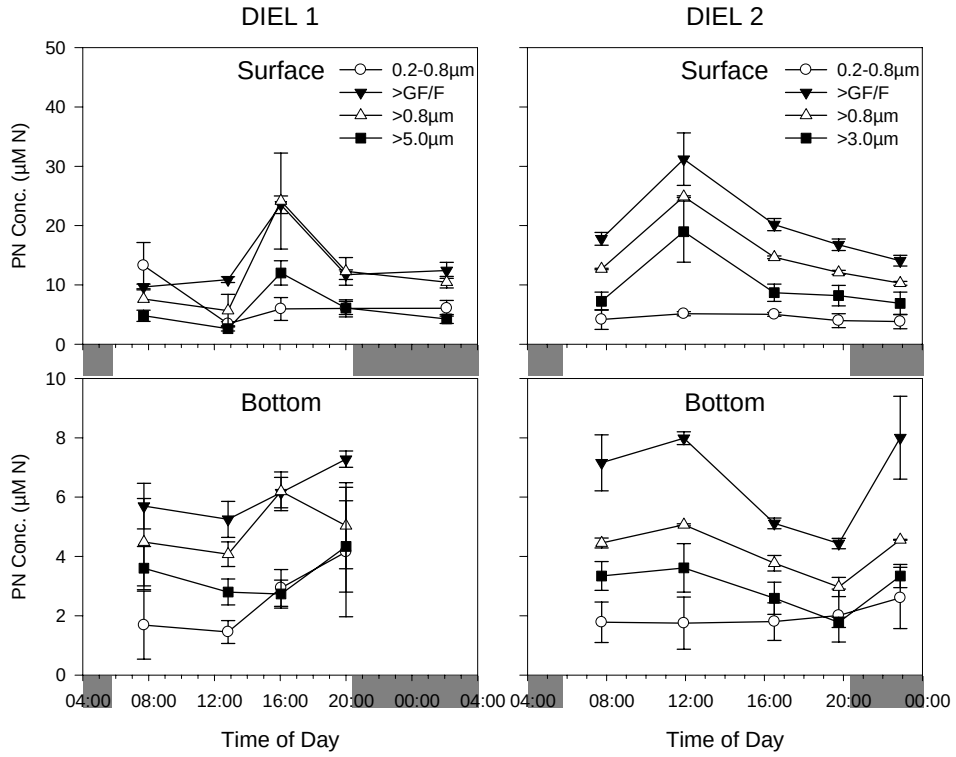


Fig. 1 Bradley et al.

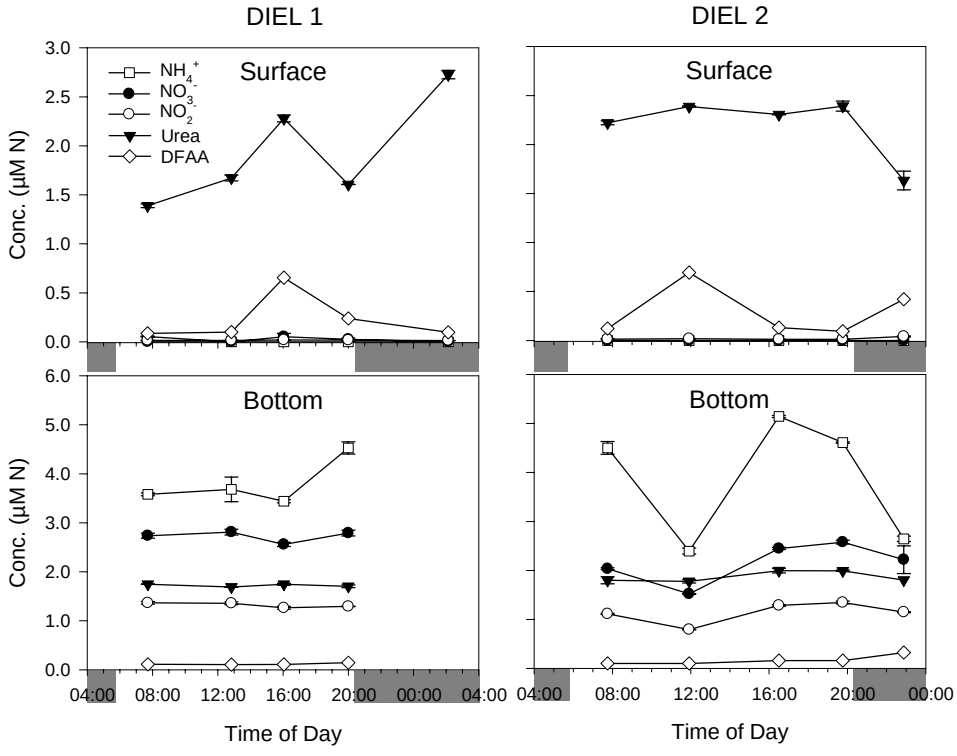


Fig. 2 Bradley et al.

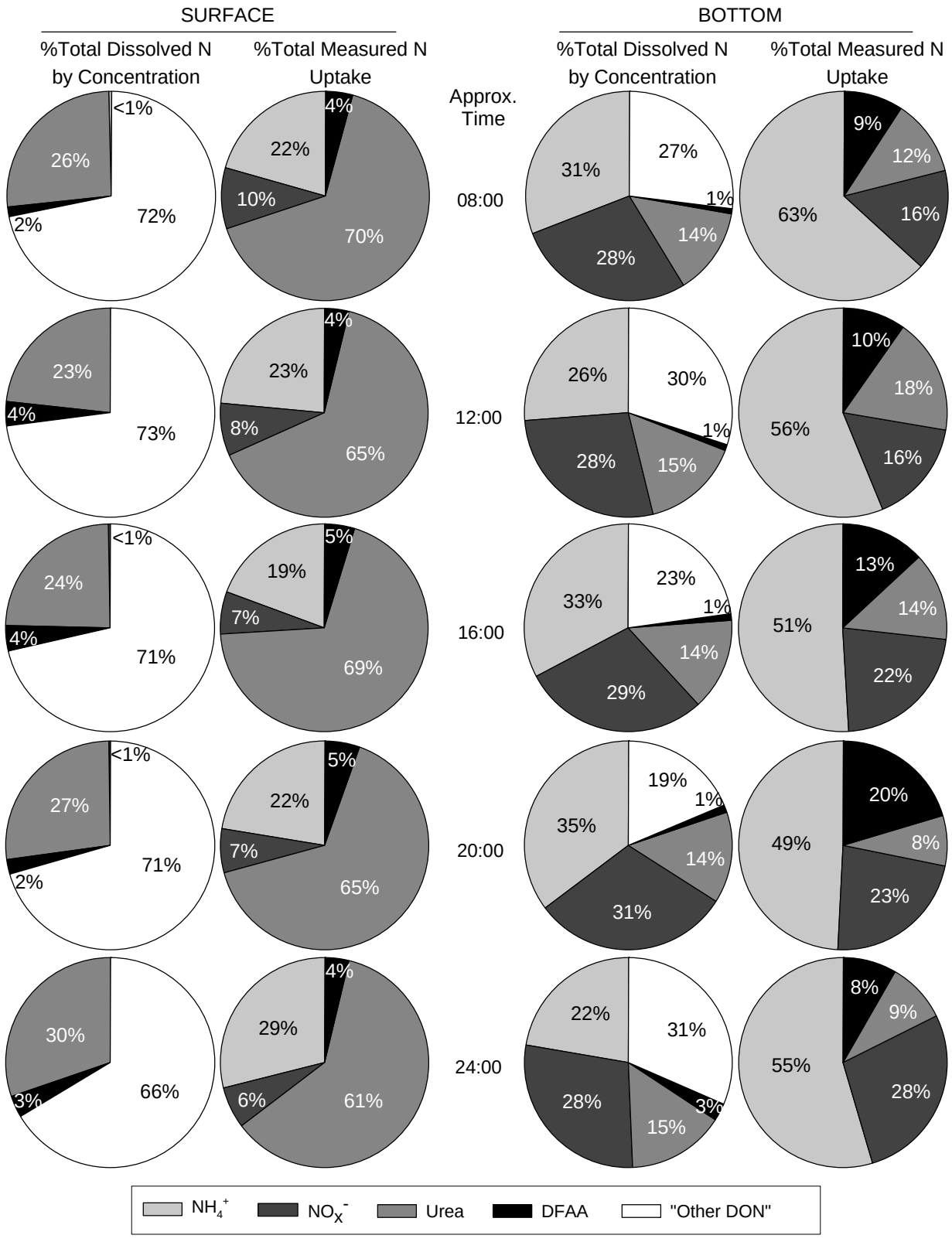
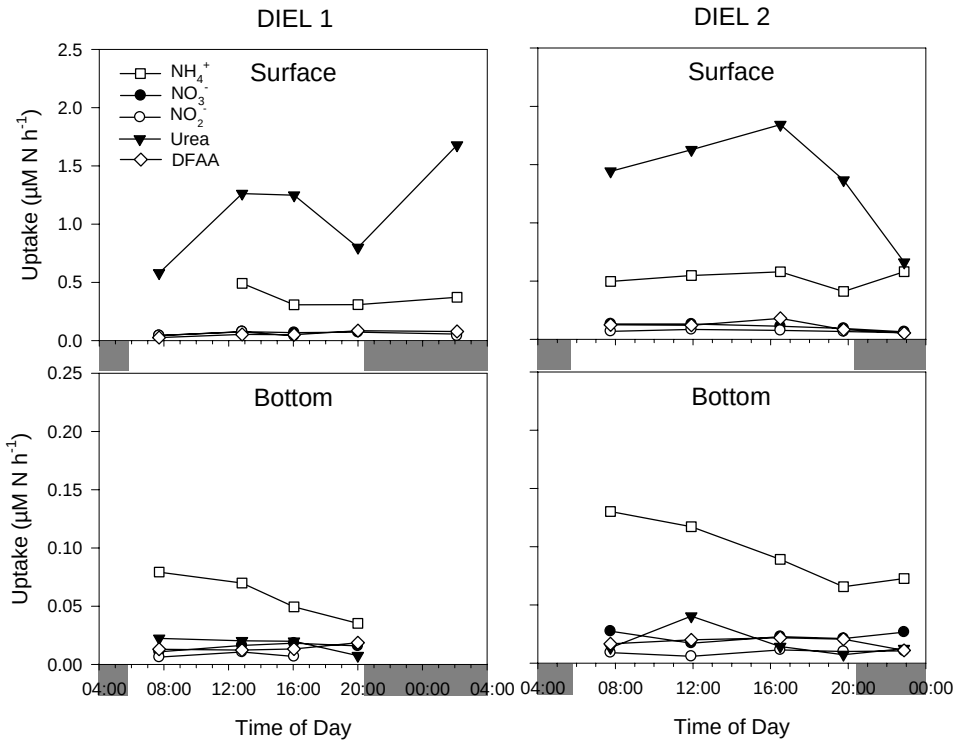


Fig. 3 Bradley et al.



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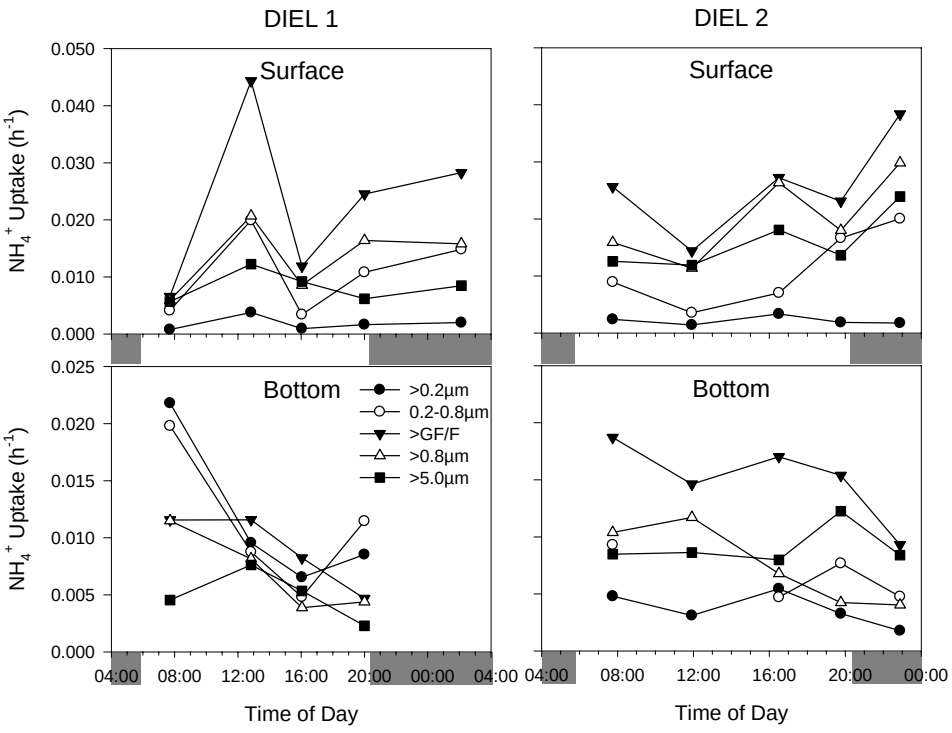


Fig. 5 Bradley et al.

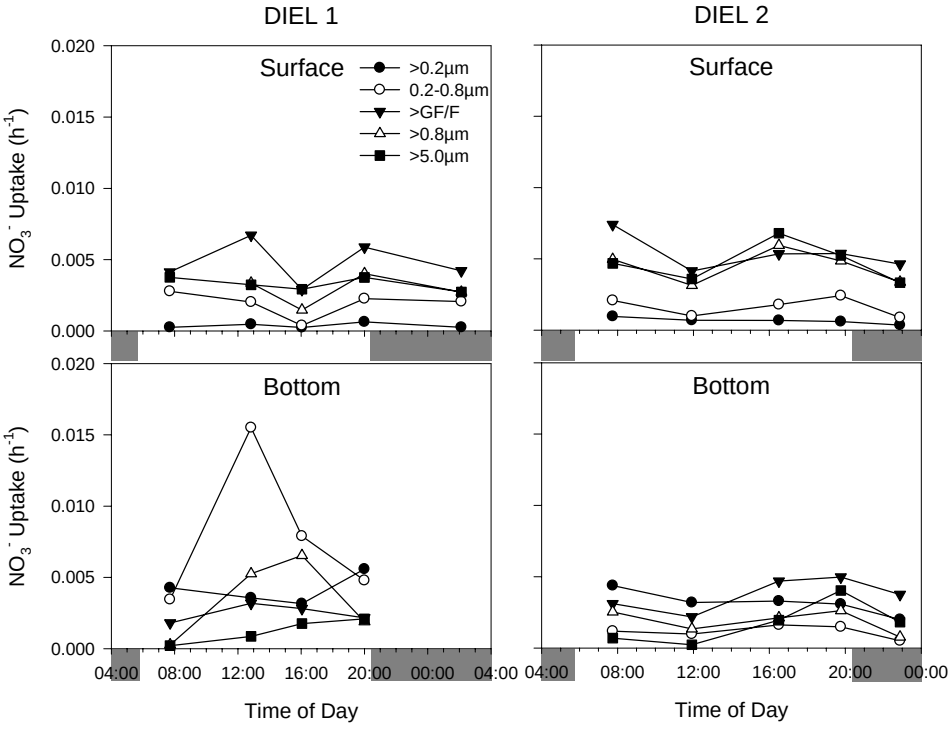


Fig. 6 Bradley et al.

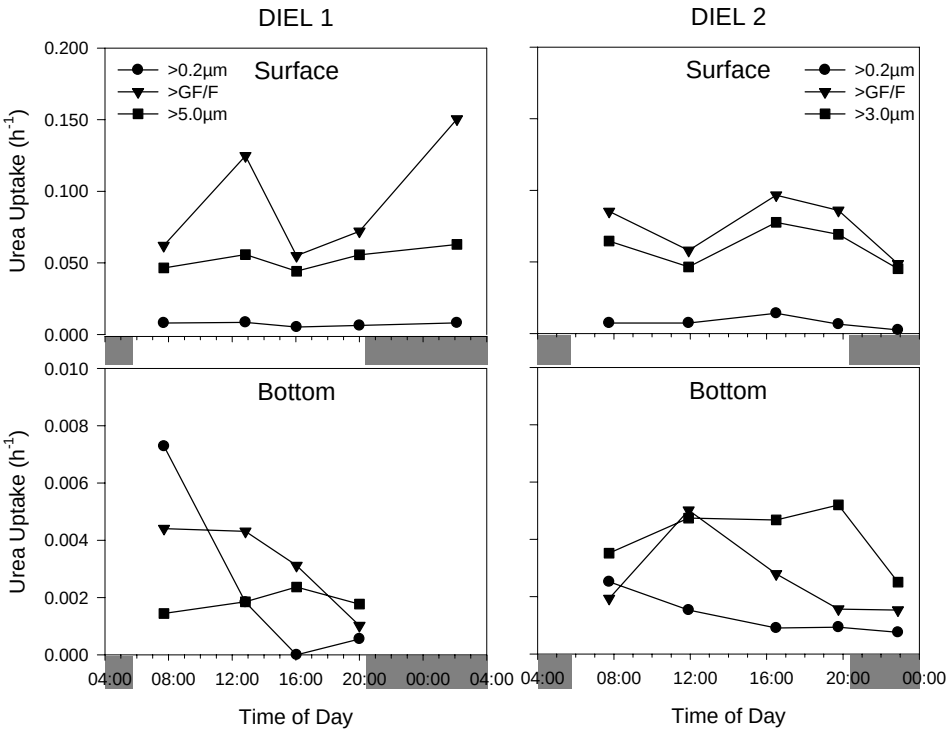
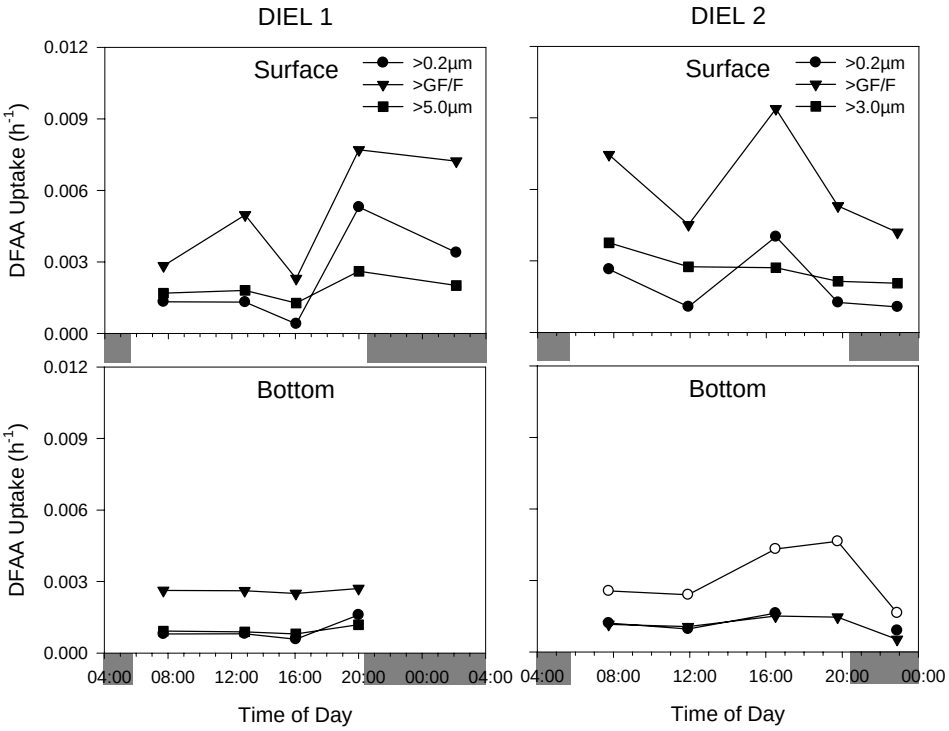


Fig. 7 Bradley et al.



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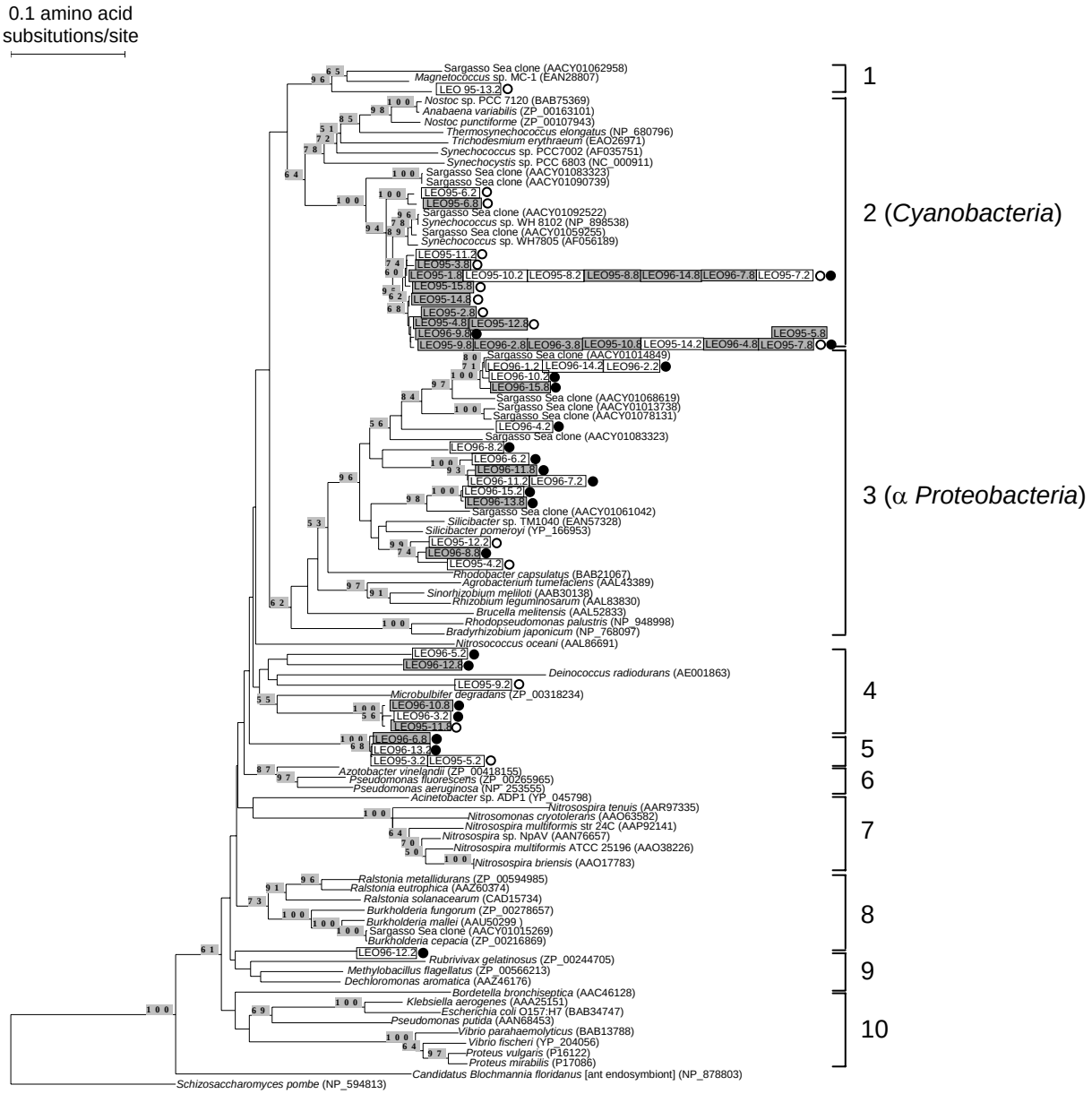

Fig. 9 Bradley et al.